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Abstract

Background: hiPSCs are generated through epigenetic reprogramming of somatic tissue. Genomic imprinting is an epigenetic phenomenon through which monoallelic gene expression is regulated in a parent-of-origin-specific manner. Reprogramming relies on the successful erasure of marks of differentiation while maintaining those required for genomic imprinting. Loss of imprinting (LOI), which occurs in many types of malignant tumors, would hinder the clinical application of hiPSCs.

Results: We examined the imprinting status, expression levels and DNA methylation status of eight imprinted genes in five independently generated hiPSCs. We found a low frequency of LOI in some lines. Where LOI was identified in an early passage cell line, we found that this was maintained through subsequent passages of the cells. Just as normal imprints are maintained in long-term culture, this work suggests that abnormal imprints are also stable in culture.

Conclusions: Analysis of genomic imprints in hiPSCs is a necessary safety step in regenerative medicine, with relevance both to the differentiation potential of these stem cells and also their potential tumorigenic properties.

Keywords: Genomic imprinting, Loss of imprinting (LOI), DNA methylation, Histone modification, Human induced pluripotent cells

Background

Human induced pluripotent stem cells (hiPSCs) represent a promising therapeutic tool for many diseases, and might be useful for regenerating aged tissues and organs at high risk of failure [1,2]. However, the intrinsic self-renewal and pluripotency of hiPSCs potentially make them tumorigenic, hindering their clinical application [3-5]. hiPSCs are generated through epigenetic reprogramming of somatic tissue. It was initially thought that hiPSCs and human embryonic stem cells (hESCs) shared a high degree of epigenetic similarity [6,7]. However, recent reports have indicated that substantial differences exist between hiPSCs and hESCs with regard to gene expression, miRNA expression and DNA methylation [8-10]. Cell-of–origin-specific genetic and epigenetic differences exist in hiPSCs [11] and some of these stem cell lines spontaneously differentiate during serial passage [12]. Extensive evaluation of hiPSCs is consequently an essential component of the process required for their safe use in regenerative medicine.

Many types of malignant tumors are characterized by complex genetic and epigenetic alterations, including loss of heterozygosity (LOH) and loss of imprinting (LOI) [13,14]. Such alterations are presumed to represent the second hit, according to Knudson's two-hit hypothesis (OMIM #167000) [15]. However, alterations in DNA methylation can also occur as the first hit during human carcinogenesis [16]. Alterations in the expression of imprinted genes represent one of the most common changes seen in cancer [17,18]. Some imprinted genes, including *H19* [19], *GTL2* [20], *PEG1*, *PEG3* [21], *LIT1* (*KCNQ1OT1*) [22] and *ZAC* [23] are known to act, or are strongly implicated to act, as

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Table 1 LOI and MOI in hiPSCs

		H19		IGF2		PEG3		PEG1		GTL2		KCNQ1		NDN		LIT1	
		(RsaI)		(ApaI)		(MnlI)		(AflIII)		(TaaI)		(SmaI)		(MboI)		(RsaI)	
Cell	Passage	gDNA	cDNA	gDNA	cDNA	gDNA	cDNA	gDNA	cDNA	gDNA	cDNA	gDNA	cDNA	gDNA	cDNA	gDNA	cDNA
AM936EP	P9	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -2	P13	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -2	P19	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -2	P35	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -3	P9	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -3	P21	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -3	P29	a/b	b	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -3	P36	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -7	P12	a/b	a	b	-	a	-	b	-	a/b	a/b	a	-	b	-	a/b	a
AM-iPS -7	P22	a/b	a	b	-	a	-	b	-	a/b	a/b	a	-	b	-	a/b	a
AM-iPS -7	P32	a/b	a	b	-	a	-	b	-	a/b	a/b	a	-	b	-	a/b	a
AM-iPS -8	P13	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -8	P20	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -8	P37	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -20	P8	N.T.	a	N.T.	-	N.T.	-	N.T.	-	N.T.	a	N.T.	-	N.T.	-	N.T.	a
AM-iPS -20	P11	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -20	P14	a/b	a	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -20	P16	a/b	N.D.	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
AM-iPS -20	P32	a/b	N.D.	b	-	a	-	b	-	a/b	a	a	-	b	-	a/b	a
PL551Ar	P16	a/b	a	a/b	a	b	-	a/b	N.D.	b	-	b	N.D.	a	-	a	-
PAE-iPS -05	P19	a/b	a	a/b	N.D.	b	-	a/b	N.D.	b	-	b	-	a	-	a	-
PAE-iPS -05	P31	a/b	a	a/b	N.D.	b	-	a/b	N.D.	b	-	b	-	a	-	a	-
PAE-iPS -11	P14	a/b	a	a/b	N.D.	b	-	a/b	N.D.	b	-	b	-	a	-	a	-
PAE-iPS -11	P18	a/b	a	a/b	N.D.	b	-	a/b	N.D.	b	-	b	-	a	-	a	-
PAE-iPS -11	P30	a/b	a	a/b	N.D.	b	-	a/b	N.D.	b	-	b	-	a	-	a	-
MRC-5	-	a/b	N.D.	b	-	a	-	b	-	a/b	a/b	a/b	N.D.	a/b	N.D.	a	-
MRC-iPS -16	P30	a/b	N.D.	b	-	a	-	b	-	a/b	ND	a/b	b	a/b	a	a	-
MRC-iPS -25	P6	a/b	N.D.	b	-	a	-	b	-	a/b	ND	a/b	b	a/b	a	a	-
MRC-iPS -25	P30	a/b	N.D.	b	-	a	-	b	-	a/b	ND	a/b	b	a/b	a	a	-
MRC-iPS -40	P11	a/b	N.D.	b	-	a	-	b	-	a/b	ND	a/b	b	a/b	a	a	-
MRC-iPS -40	P30	a/b	N.D.	b	-	a	-	b	-	a/b	ND	a/b	b	a/b	a	a	-
UtE1104	P9	a/b	N.D.	a	-	a/b	a/b	a/b	b	a/b	a/b	b	N.D.	b	-	a	-
UtE-iPS -6	P20	a/b	N.D.	a	-	a/b	a/b	a/b	a/b	a/b	a/b	b	-	b	-	a	-
UtE-iPS -6	P31	a/b	b	a	-	a/b	a/b	a/b	a/b	a/b	a/b	b	-	b	-	a	-
UtE-iPS -11	P13	a/b	N.D.	a	-	a/b	N.D.	a/b	a/b	a/b	a	b	N.D.	b	-	a	-
UtE-iPS -11	P20	a/b	N.D.	a	-	a/b	N.D.	a/b	a/b	a/b	a/b	b	N.D.	b	-	a	-
UtE-iPS -11	P30	a/b	N.D.	a	-	a/b	N.D.	a/b	a/b	a/b	a	b	-	b	-	a	-
Edom22	P5	b	-	a/b	a/b	a	-	a/b	b	b	-	a/b	a	a	-	a	-
Edom-iPS -1	P27	b	-	a/b	N.D.	a	-	a/b	b	b	-	a/b	a	a	-	a	-

Table 1 LOI and MOI in hiPSCs *(Continued)*

hES 3	P29	a/b	a	b	-	a/b	a	a/b	b	b	-	a	-	a/b	b	a	-
SEES 1	P10	a/b	a	a/b	a	a	-	b	-	a/b	b	a	-	a	-	a	-
SEES 4	P9	a/b	b	a/b	a	a	-	b	-	b	-	a	-	a/b	b	a	-

A summary of LOI and MOI RFLP data for the 8 imprinted genes analyzed in 22 hiPSCs and 3 control hES cell lines. hiPSCs derived from extraembryonic amniotic membrane (AM-iPS), embryonic lung tissue (MRC-iPS), uterine endometrium (UtE-iPS), adult menstrual blood (Edom-iPS) and extraembryonic placental tissue (PAE-iPS). Samples were analyzed at the specified passage number. (-): not informative.

tumor suppressor genes (TSGs). Furthermore, imprinted genes play key roles in regulating growth and differentiation [24]. Thus the aberrant expression of imprinted genes may contribute to tumorigenesis or alter the differentiation potential of stem cells.

The monoallelic expression of imprinted genes is reliant on epigenetic mechanisms, most notably DNA methylation, which is established in the male and female germlines at discrete locations termed germline or gametic differentially methylated regions (gDMRs) [25]. Imprinted domains generally contain several genes displaying allele-specific expression and gDMRs within these domains act as imprinting centers or imprint control regions for the domain [26]. The majority of imprinted genes reside within these complex domains [27]. Although gametic DMRs are maintained throughout the life of the organism,

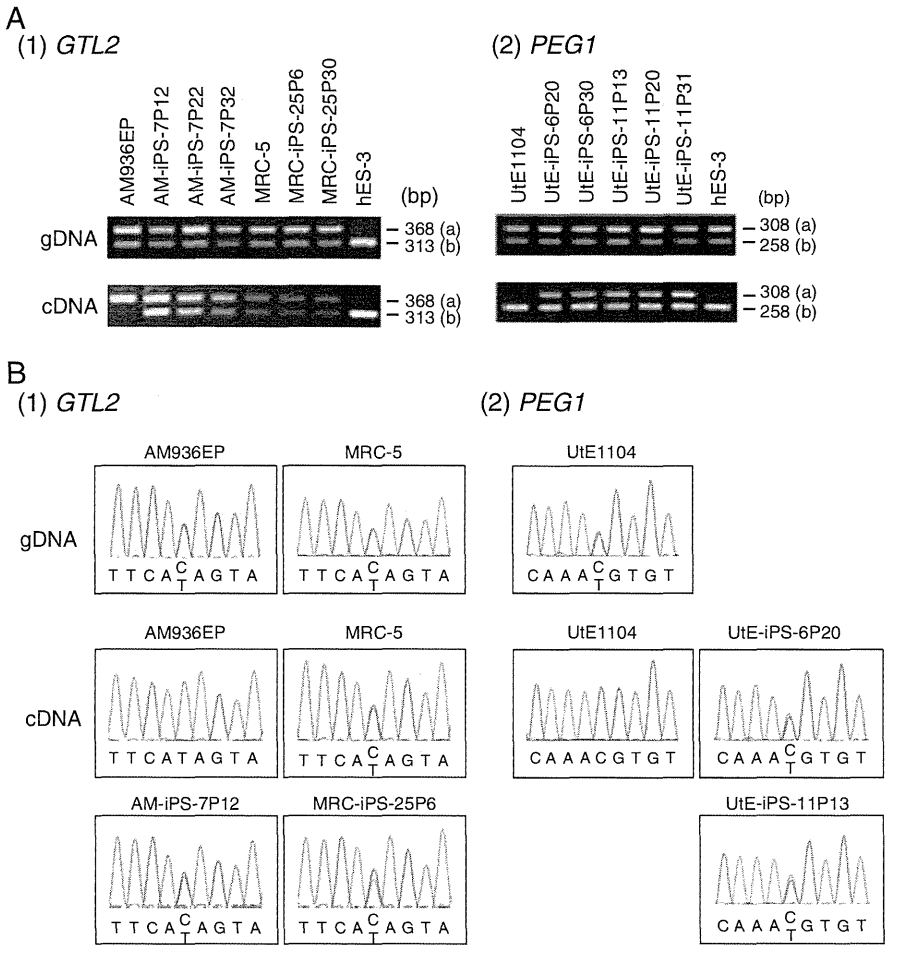

Figure 1 Loss of imprinting in hiPSCs. Loss of allelic expression of *GTL2* in cell line AM-iPS-7 (1) and *PEG1* in cell lines UtE-iPS-6 and 11 (2). PCR products amplified from paired genomic DNA and cDNA samples were digested with the specified restriction enzymes (**A**). Results were confirmed by direct sequencing (**B**). AM936EP and UtE1104 are primary culture cells made from amniotic membrane and uterine endometrium cells, respectively. Band 'a': without a restriction site. Band 'b': with a restriction site.

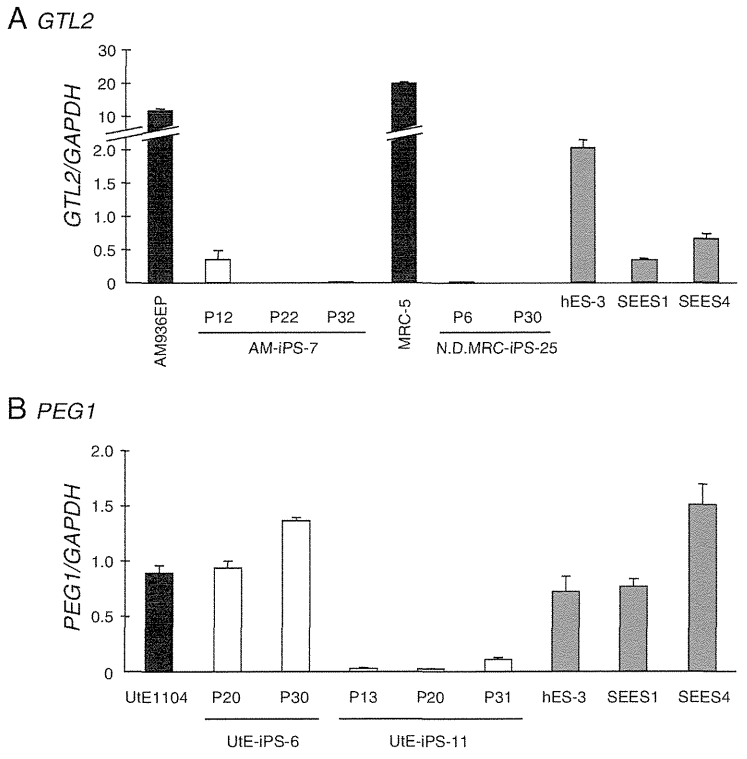

Figure 2 Reduced expression of hiPSCs with LOI. Gene expression levels of *GTL2* (**A**) and *PEG1* (**B**) in the original and the passaged hiPSCs were compared to that of hESCs. The *GAPDH* ratio was calculated. The bars indicate the means ± standard deviation (SD) from two replicates. N.D.: not determined.

genes within the domain can be imprinted in tissue- and developmentally specific manners [28].

In a recent paper, we demonstrated that hiPSCs exhibit epigenetic patterns distinct from hESCs [29]. After continuous passaging of the hiPSCs, these differences diminished such that over time the hiPSCs more closely resembled hESCs. However, we found that the imprinted DMRs showing abnormal methylation in early passage hiPSCs did not resolve during passaging. In this study we focused on the expression of imprinted genes in hiPSCs. Several reports on imprinted gene expression in hESCs demonstrate a substantial degree of instability [30]. Less is known regarding the stability of imprints in hiPSCs, although some work has begun [31]. We are particularly concerned with the stability of imprints in pluripotent stem cells during prolonged culture. Here, we examined the imprinting status and expression levels of eight imprinted genes and the methylation status of their DMRs in five independently derived hiPSCs. We found that the frequency LOI was very low in the early passaged lines. We also found that, in contrast, the epigenetic changes that took place at non-imprinted loci during prolonged culture for both normal and aberrant imprints were stably inherited despite prolonged passaging of the lines.

Results

Loss of heterozygosity (LOH) and loss of imprinting (LOI) in hiPSCs

We first determined whether hiPSCs showed LOH by comparing the restriction fragment length polymorphism (RFLP) patterns of the original tissue DNA with those of the hiPSC DNA samples. Samples in which RFLPs were present in the original DNA sample but absent or with an altered ratio in the hiPSC samples were considered to exhibit LOH. We found no evidence for LOH at the 8 loci tested (*H19*, *IGF2*, *KCNQ1*, *LIT1*, *GTL2*, *PEG1*, *PEG3* and *NDN*).

We next performed RT-PCR and RFLP analyses to identify samples that demonstrated loss of imprinting (LOI). Where expression of genes was low in undifferentiated cells, it was not possible to determine their imprinting status (*H19* in MRC-iPS and UtE-iPS, *IGF2* in PAE-iPS and *GTL2* in Edom-iPS). Of the 16 informative loci, we identified LOI at three loci in hiPSCs, *GTL2*, *PEG1* and *PEG3*, but we did not detect any LOI in hESCs (Table 1). Of particular interest, we observed loss of imprinting during the

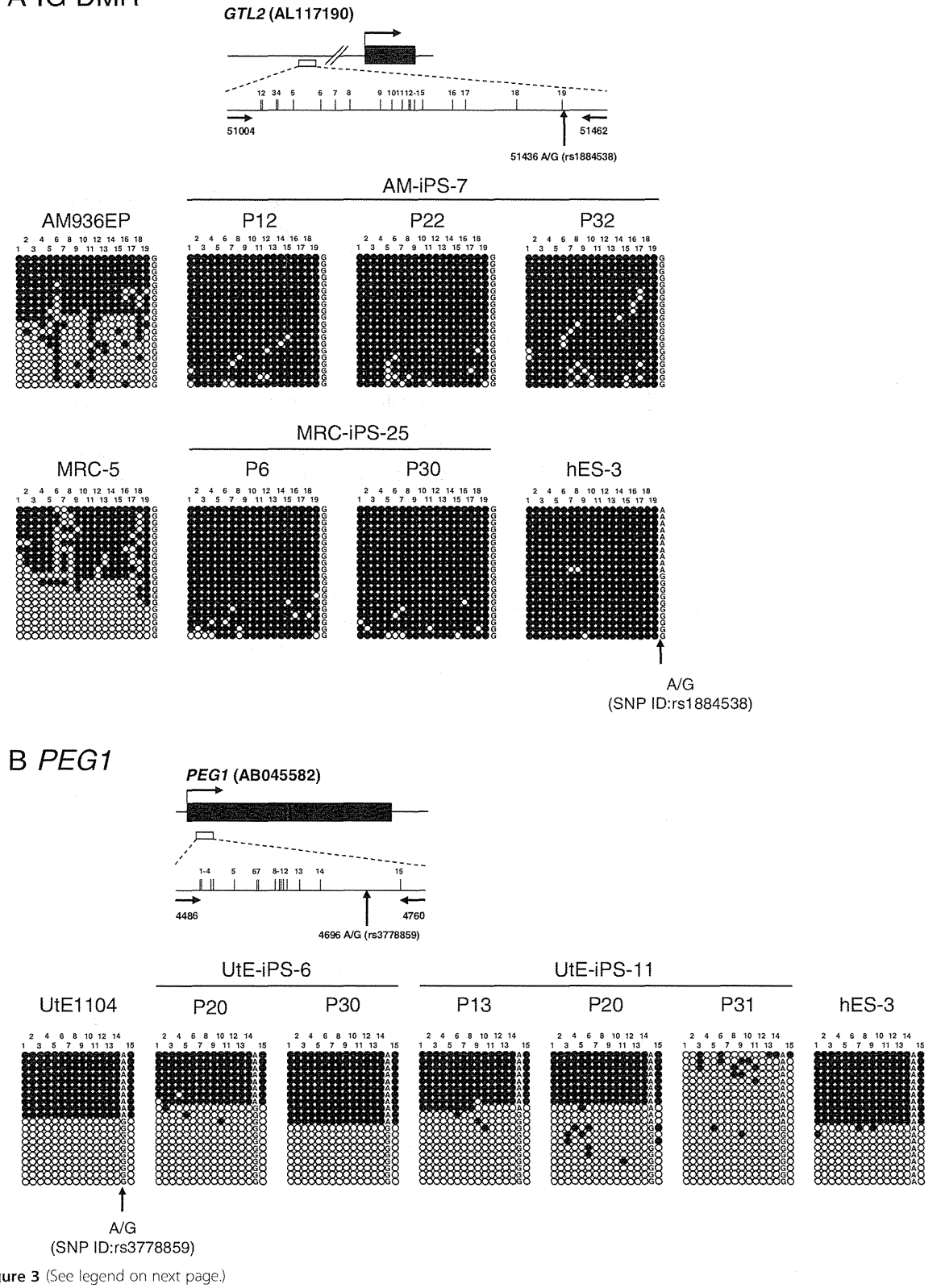

Figure 3 (See legend on next page.)

(See figure on previous page.)
Figure 3 Aberrant DNA methylation of hiPSCs with LOI. Bisulfite PCR sequencing methylation assay of genomic DNA prepared from AM-iPS-7 and MRC-iPS-25 at the IG-DMR (*GTL2*-DMR) (**A**) and UtE-iPS-6 and 11 at *PEG1* (**B**). Each row represents a unique methylation profile within the pool of 20 clones sequenced. Closed and open circles represent methylated and unmethylated CpGs, respectively. The numbers represent the percentages of methylation by bisulfite sequencing. SNPs are shown by arrows.

process of establishing the AM-iPSC (*GTL2)* and UtE-iPSC (*PEG1*) lines (Figure 1A). Where LOI was observed in early passage cells, this was maintained even after 30 or more passages (Figure 1).

Expression level of the imprinted genes in hiPSCs

LOI can refer to silencing of an originally active allele or expression of a normally silent allele. Therefore, we compared the expression levels of the three genes that displayed LOI in hiPSCs and hESCs (Table 1). The expression of *IGF2* and *GTL2* was decreased in almost all the hiPSC lines in comparison with the hES cells (Additional files 1 and 2). *GTL2* in cell line AM-iPS-7 and *PEG1* in line UtE-iPS-11 showed apparent biallelic expression but their expression levels were relatively low in comparison to hESCs with reduced expression maintained stably through to late passages (Figure 2). In contrast, expression of *PEG1* in cell line UtE-iPS-6 was not significantly different from that of hESCs. These results were in accordance with the DNA microarray analysis data we already reported [29]. Since, in two cases, LOI correlated with reduced gene expression, this has potential functional implications due to loss of function.

Analysis of the DNA methylation status and the histone modification of GTL2 and PEG1 DMRs in hiPSC lines

We determined the allele-specific methylation status of the *GTL2* (IG-DMR) and *PEG1* imprinted DMRs using polymorphic bisulfite-PCR sequencing (Figure 3). In cell line AM-iPS-7, which showed LOI and reduced expression of *GTL2,* we observed hypermethylation of IG-DMR, which was maintained during continuous passaging. IG-DMR methylation is normally present on the silent allele of *GTL2* [32], which suggests aberrant signaling between this DMR and *GTL2* expression. In cell line UtE-iPS-11, in which there was LOI and reduced expression of *PEG1,* abnormal methylation was detected in passage 31 cells but not earlier passages. In cell line UtE-iPS-6, in which there was LOI but not reduced expression of *PEG1,* abnormal methylation was not detected. Allele-specific expression of some genes has been reported to be regulated by histone modification rather than direct DNA methylation [33-35]. We therefore analyzed histone modifications in the hiPS cell line by chromatin immunoprecipitation (ChIP) analyses using the following antibodies: dimethylated H3-Lys4 (H3K4me2), acetylated H3-Lys9 (H3K9ac), H3K9me2, and H3K27me3. H3K4me2 and H3K9ac mark active genes and H3K9me2 and H3K27me3 are repressive marks. In the *GTL2* promoter region, H3K9me2 and H3K27me3 were enriched in AM-iPS-7 and MRC-iPS-25 cells (Figure 4D).

Reactivation of the imprinted genes by the HDACi treatment

Previous reports demonstrated that the *GTL2* gene was aberrantly silenced in most mouse iPSC lines but that expression could be restored through HDACi treatment [36,37]. In our study, AM-iPS and MRC-iPS cells showed LOI of *GTL2,* with a reduction in gene expression and hypermethylation of the IG-DMR. To assess whether *GTL2* expression could be restored, AM-iPS and MRC-iPS cells were treated with the HDAC inhibitor VPA (sodium valproate). VPA-treated cells did achieve a 3.0–5.8-fold increase in *GTL2* expression levels (Figure 4A) and H3K4me2 and H3K9ac were enriched in its promoter region (Figure 4D). However, the DNA methylation pattern was stable under VPA treatment and the imprinting status of *GTL2* was not changed, with cells maintaining biallelic expression of the gene (Figure 4B). These results suggested that the aberrant DNA methylation and imprinting that were established and maintained in early passages (Figure 4C) were not sustainably reversed by the treatment.

Discussion

Most hES and hiPS cell lines possess stable imprinted gene expression, at least in undifferentiated cells [30,31 and findings in this study]. This implies that imprints withstand the process of reprogramming and the rigors of growing in culture. In our study, we found that only three of the 22 hiPS cell lines we derived from a variety of somatic cell types showed LOI, and at only a few sites. The majority of cases had normal imprinting status. While LOI was rare in our hiPS cell lines, we found that it was maintained during prolonged passage, and resistant to VPA treatment. These abnormalities would preclude the use of these cell lines for therapeutic applications but might provide a mechanistic insight relevant to imprinting and reprogramming.

We previously reported that abnormal DNA methylation detected in early passage iPSCs diminished after continued passaging, such that these cells ultimately more resembled ESCs. However, abnormal DNA methylation at imprinted loci in ESCs occurs in response to continuous passaging [29]. Rugg-Gunn et al. suggested three possible explanations for LOI in hESCs [30]. First, the developmental onset of transcription might influence imprinted gene expression. Second, a particular imprinted gene's expression might

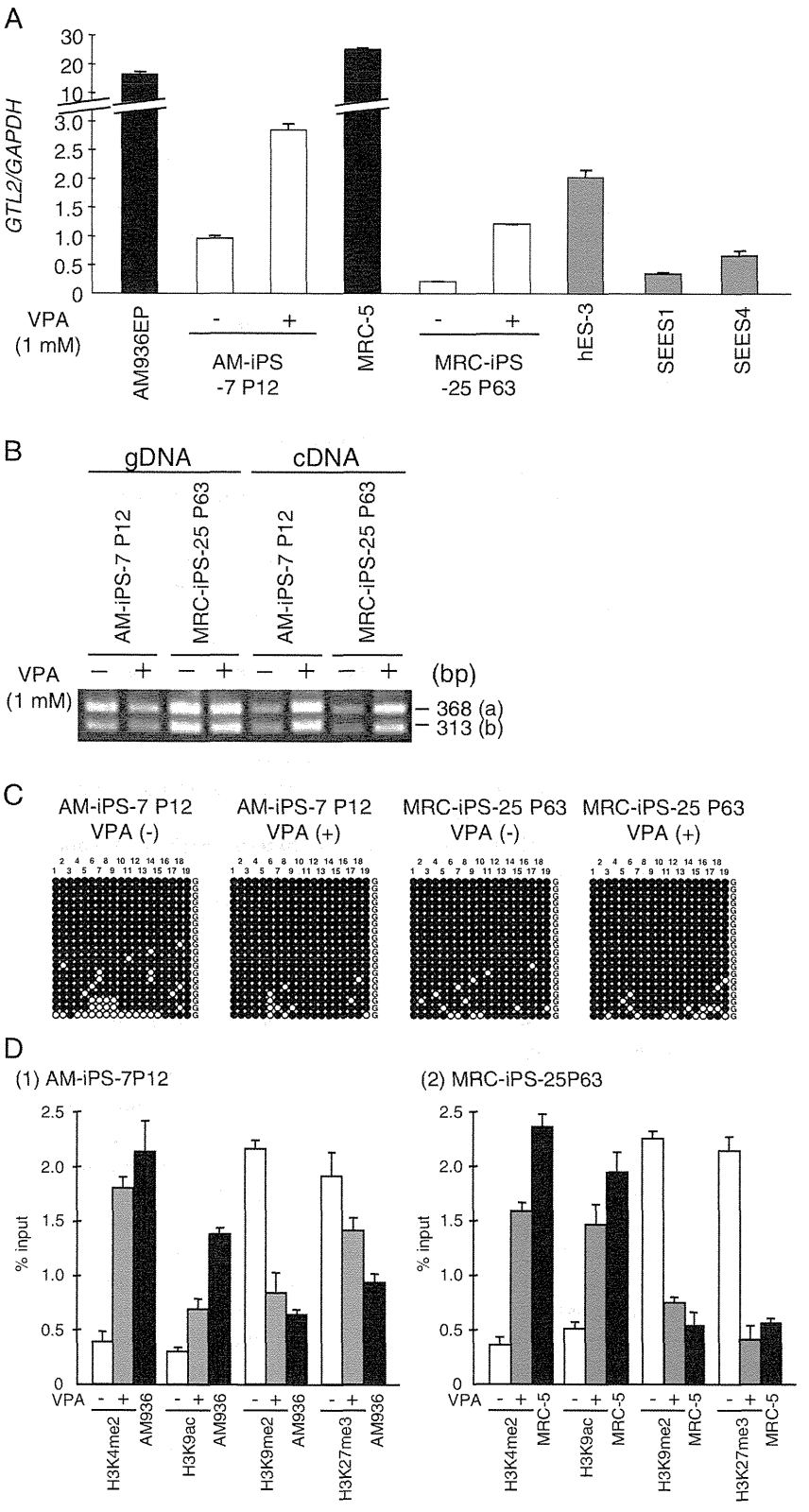

Figure 4 (See legend on next page.)

(See figure on previous page.)
Figure 4 Reactivation of *GTL2* expression by treatment with VPA. Reactivation of *GTL2* expression by treatment with VPA (**A**). The expression level of *GTL2* mRNA was restored by VPA treatment. Gene expression of the original cells and of the hiPSCs was compared to that of hESCs. The *GAPDH* ratio was calculated. The bars indicate the means ± SD from two replicates. The imprinting status of *GTL2* was stable in response to VPA (**B**). Methylation status in bisulfite-PCR sequencing analyses of IG-DMR is unchanged (**C**). Histone modifications of the *GTL2* promoter were changed by VPA (D). The immunoprecipitation/input ratio was calculated. The bars indicate the means ± SD from three replicates.

differ depending on whether it is regulated by maternally or paternally inherited methylation. Third, the pattern of imprinted gene expression might depend on whether the gene provides a growth advantage to hESCs. These possibilities might also apply to hiPSCs.

There are two caveats that apply to this work. First, we examined expression in undifferentiated cells. Consequently, we may have missed changes in imprinted gene expression where genes are expressed only in differentiated cells or where imprinting is tissue specific. Second, we examined total levels of expression and total methylation patterns of populations of cells. Therefore we cannot exclude the possibility that a small population within our samples could behave in a different manner from the general population. Nonetheless, our data are encouraging in suggesting that imprinting errors in iPSCs are derived from a variety of human somatic cell types.

One of the key advantages of iPSCs is that they can be derived from patients, supporting the further investigation of certain diseases, as well as the replacement of degenerated and damaged tissues. Careful analysis of imprinted genes should therefore be performed on all iPS cell lines since several published iPS cell lines that passed the necessary reprogramming criteria also showed aberrations in imprinted gene expression and DNA methylation of DMRs. This is particularly critical if these hiPSs are to be used for regenerative medicine since aberrations in imprinted genes could cause problems with cell differentiation and perhaps even cause tumors [38]. The analysis of imprinted genes is also essential for modeling of genetic diseases because abnormal imprinting can seriously confuse the disease phenotyping.

Recent advances in high-throughput technologies for gene expression analysis and DNA methylation analysis indicate the possibility that all newly generated stem cell lines can be characterized at the epigenetic level rapidly and precisely. However, our work and that of others suggest that certain imprinted loci may be more susceptible to LOI. This means that it might be possible to design targeted assays for specific loci as the first step in the characterization of newly generated cell lines, and also those that have been extensively passaged.

Conclusions

In conclusion, while imprinting errors may be rare in iPSCs, they are resistant to reversal strategies. The aberrant expression of imprinted genes in these lines is likely to hamper their use both for the understanding of certain pathologies and regenerative medicine.

Methods

Ethics statement

All experiments handling human cells and tissues were performed in line with the tenets of the Declaration of Helsinki. This study was approved by the Institutional Review Board of the National Institute for Child Health and Development and the Ethics Committee of Tohoku University School of Medicine.

DNA/RNA preparation of iPSCs

We generated 22 hiPSCs from extraembryonic amniotic membrane (AM-iPS), embryonic lung tissue (MRC-iPS), uterine endometrium (UtE-iPS), adult menstrual blood (Edom-iPS), and extraembryonic placental tissue (PAE-iPS) and characterized the pluripotent nature using culture methods described previously [39-41]. Prior to RNA and DNA preparation, feeder layers were removed from the undifferentiated cells by panning for 20 minutes.

Loss of heterozygosity (LOH) and loss of imprinting (LOI) analyses

PCR was performed on parental tissue and the genomic DNA of hiPSCs using the primer sequences summarized in Additional file 3. A PCR reaction mix containing 0.5 μM concentrations of each primer set, 200 μM dNTPs, 1× PCR buffer, and 1.25U of EX *Taq* Hot Start DNA Polymerase (Takara Bio, Tokyo, Japan) in a total volume of 20 μl was used. The following PCR program was used: 1 minute of denaturation at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 50-70°C, 30 seconds at 72°C and a final extension for 5 minutes at 72°C. PCR products were digested by unique polymorphic enzymes to identify samples that were heterozygous for a single nucleotide polymorphism (SNP). For samples found to be heterozygous for a SNP, RNA was prepared from matched hiPSCs, followed by reverse transcription-PCR (RT-PCR) and restriction digestion (Additional file 3) [42-49]. The digested PCR products were electrophoresed on 3% agarose gel.

Gene expression analysis

RNA expression levels of 8 imprinted genes were also analyzed by microarray and the real-time PCR. Microarray analysis was performed using an Agilent Whole Human

Genome Microarray chip (G4112F, Agilent, Santa Clara, CA). Raw data were normalized and analyzed using GeneSpringGX11 software (Silicon Genetics, Redwood City, CA). The microarray data have been deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Real-time PCR reaction was done with SYBR Premix Ex Taq II (Takara Bio). In the case of PEG3 expression analysis, TaqMan Gene Expression Assay (Assay ID: Hs00300418-s1, Applied Biosystems, Foster City, CA) was carried out according to the manufacturer's protocol using a StepOne Real-time PCR System (Applied Biosystems). The relative expression levels of the detected genes from these cells were estimated visually by comparing relative band intensities with the expression level of the housekeeping gene *GAPDH*.

Polymorphic bisulfite PCR methylation assay

We performed standard methylation assays using the SNPs and bisulfite sequencing [50]. The primary DMRs of eight imprinted genes (*H19, GTL2, ZDBF2, PEG1, KCNQ1OT1, ZAC, PEG3* and *SNRPN*) were analyzed as described previously [50,51]. Each DNA sample was treated with sodium bisulfite using the EZ methylation kit (Zymo Research, Orange, CA) and amplified by PCR. PCR products were purified, cloned into pGEM-T (Promega, Medison, WI) and an average of 20 clones per individual were sequenced using reverse primer M13 and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). To avoid any allelic bias, we used specific polymorphic sites. Sodium bisulfite modification treatments were carried out in duplicate for each DNA sample and at least three independent amplification experiments were performed for each individual examined.

Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed using the Magna ChIP G Chromatin Immunoprecipitation Kit (Millipore, Temecula, CA) according to the manufacturer's protocol. We used the following antibodies: dimethylated H3-Lys4, acetylated H3-Lys9, dimethylated H3-Lys9 and trimethylated H3-Lys27 (Millipore). The histone modifications were analyzed by real-time PCR. Real-time PCR reaction was done with SYBR Premix Ex Taq II (Takara Bio). The amount of precipitated DNA was determined as percentage relative to input DNA. Primers used are listed in Additional file 3.

Treatment of cells with sodium valproate

hiPSCs were plated at a density of 5×10^5 cells/60mm^2 dish. Twenty-four hours later, they were treated with 1 mM sodium valproate (Wako, Tokyo, Japan) for the times stated. Total RNA was prepared and analyzed by the RT-PCR method. The methylation status of the DMRs was examined using the bisulfite PCR sequencing methylation assay described previously [51].

Additional files

Additional file 1: Microarray analysis. Scatter plots of MRC-iPS-25P22 versus hES3 (**A**) and UtE-iPS-6P30 versus hES3 (**B**). Scatter plot comparing the spot intensities in hybridization with probes from hiPSCs (*y* axis) and hESCs (*x* axis). The magenta plots indicate the imprinted genes.

Additional file 2: Gene expression analysis of the imprinted genes. *H19* (**A**), *IGF2* (**B**), *PEG3* (**C**), *PEG1* (**D**), *GTL2* (**E**), *KCNQ1* (**F**), *NDN* (**G**) and *LIT1* (**H**). Gene expression of the original and hiPSCs was compared to that of hESCs. The *GAPDH* ratio was calculated. The bars indicate the means ± SD from two replicates.

Additional file 3: PCR primers and conditions.

Abbreviations

DMR: Differentially methylated region; hESCs: Human embryonic stem cells; hiPSCs: Human induced pluripotent cells; LOH: Loss of heterozygosity; LOI: Loss of imprinting; MOI: Maintenance of imprinting; N.D: Not determined; N.T: Not tested; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; RT-PCR: Reverse transcription-PCR; SNP: Single nucleotide polymorphism; SD: Standard deviation.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

AU and TA conceived and designed the study and wrote the manuscript. AU generated hiPSCs and hESCs. TA analyzed genomic imprinting. HH carried out the molecular study and data analysis and wrote the manuscript. H Okae, MS, NM and AS performed the molecular study and contributed to data analysis. MT, NK, H Okita, YM and HA prepared cell materials and contributed to data analysis. KN performed the transcriptome data analysis. All authors reviewed the results from the data analysis and approved the final manuscript.

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Podocalyxin Is a Glycoprotein Ligand of the Human Pluripotent Stem Cell-Specific Probe rBC2LCN

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EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM (iPS) CELLS

Podocalyxin Is a Glycoprotein Ligand of the Human Pluripotent Stem Cell-Specific Probe rBC2LCN

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ABSTRACT

In comprehensive glycome analysis with a high-density lectin microarray, we have previously shown that the recombinant N-terminal domain of the lectin BC2L-C from *Burkholderia cenocepacia* (rBC2LCN) binds exclusively to undifferentiated human induced pluripotent stem (iPS) cells and embryonic stem (ES) cells but not to differentiated somatic cells. Here we demonstrate that podocalyxin, a heavily glycosylated type 1 transmembrane protein, is a glycoprotein ligand of rBC2LCN on human iPS cells and ES cells. When analyzed by DNA microarray, podocalyxin was found to be highly expressed in both iPS cells and ES cells. Western and lectin blotting revealed that rBC2LCN binds to podocalyxin with a high molecular weight of more than 240 kDa in undifferentiated iPS cells of six different origins and four ES cell lines, but no binding was observed in either differentiated mouse feeder cells or somatic cells. The specific binding of rBC2LCN to podocalyxin prepared from a large set of iPS cells (138 types) and ES cells (15 types) was also confirmed using a high-throughput antibody-overlay lectin microarray. Alkaline digestion greatly reduced the binding of rBC2LCN to podocalyxin, indicating that the major glycan ligands of rBC2LCN are presented on *O*-glycans. Furthermore, rBC2LCN was found to exhibit significant affinity to a branched *O*-glycan comprising an H type 3 structure (K_a, $2.5 \times 10^4\ M^{-1}$) prepared from human 201B7 iPS cells, indicating that H type 3 is a most probable potential pluripotency marker. We conclude that podocalyxin is a glycoprotein ligand of rBC2LCN on human iPS cells and ES cells. STEM CELLS TRANSLATIONAL MEDICINE *2013;2:265–273*

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INTRODUCTION

Human induced pluripotent stem (iPS) cells and embryonic stem (ES) cells with characteristics of self-renewal and pluripotency are attractive sources of cells for cell-replacement therapies. Before pluripotent stem cells can be used clinically, however, an important safety concern to be addressed is that residual undifferentiated cells could form tumors in patients. The use of cell surface pluripotency markers would serve as a standard and indeed a powerful technique to detect, and ideally remove, such teratoma-forming undifferentiated cells. Monoclonal antibodies that include anti-SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 have been used as probes for this purpose [1–3]. Among them, SSEA-3 and SSEA-4 are carried by globo-series glycolipids, consisting of Galβ1–3GalNAcβ1–3Galα1–4Galβ1–4Glc-Cer and its α2–3-sialylated form (Siaα2–3Galβ1–3GalNAcβ1–3Galα1–4Galβ1–4Glc-Cer), respectively. On the other hand, TRA-1-60 and TRA-1-81 markers have been reported to be expressed on podocalyxin, a heavily glycosylated membrane protein [4]. Their carbohydrate epitopes were first reported to be keratan sulfate but were recently identified by glycan microarray analysis to be type 1 *N*-acetyllactosamine (Galβ1–3GlcNAc) [5]. More recently, anti-SSEA-5 was proposed as a novel antibody probe to detect as well as remove human iPS cells and ES cells through its specificity to H type 1 glycan (Fucα1–2Galβ1–3GlcNAc), although its carrier protein has not been identified [6]. It should be noted that all of these cell surface pluripotency markers are "carbohydrate antigens" that have been known to reflect the degree of cell differentiation/undifferentiation [3].

To understand the cellular glycome of human iPS cells and ES cells in a comprehensive way, we previously performed glycome analysis of a large set of human iPS cells (114 cell types) and ES cells (9 cell types) using an advanced high-density lectin microarray [7]. In that study, the expression of α2–6Sia, α1–2Fuc, and type 1 LacNAc was strongly indicated to be increased upon induction of pluripotency [7], and consistent with this, responsible glycosyltransferase genes, that is,

ST6Gal1/2, *FUT1/2*, and *B3GalT5*, involved in the synthesis of these glycan epitopes showed significant increases in expression levels [7]. Furthermore, we found that a lectin designated rBC2LCN (recombinant N-terminal domain of BC2L-C), which was originally derived from *Burkholderia cenocepacia*, bound all of the undifferentiated cells tested, but not at all to any of the differentiated cells [7, 8]. Notably, this lectin was shown to have a strong preference to bind the defined epitope structure Fucα1–2Galβ1–3GlcNAc/GalNAc (H type 1/3/4), which comprises two of the above characteristics of pluripotency, that is, α1–2Fuc and type 1 LacNAc [7].

rBC2LCN is a small protein with a molecular weight of 15 kDa having a compact jellyroll architecture composed of 11 β strands and a short α helix, similar to the structure of tumor necrosis factor-like proteins [8]. rBC2LCN can be expressed at high levels in a soluble form in the cytoplasm of *Escherichia coli* (up to 80 mg/l) and can be purified to homogeneity in a one-step sugar-immobilized affinity chromatography approach [7]. rBC2LCN is highly specific to the defined glycan epitope Fucα1–2Galβ1–3GlcNAc/GalNAc, which is contained in glycans such as H type 1 (*Fucα1–2Galβ1–3GlcNAc*), H type 3 (*Fucα1–2Galβ1–3GalNAc*), Le^b (*Fucα1–2Galβ1–3*(Fucα1–4)*GlcNAc*), and Globo H (*Fucα1–2Galβ1–3GalNAc*β1–3Galα1–4Galβ1–4Glc) [8]. Therefore, rBC2LCN, unlike antibody, could serve as a novel type of detection reagent of pluripotent stem cells [9], particularly given that it is cost-effective and easy to produce in large amounts.

Here we demonstrate that podocalyxin, previously known as a Tra-1-60/81 carrier protein, functions as the glycoprotein ligand of the human pluripotent stem cell-specific probe rBC2CLN on undifferentiated iPS cells and ES cells [10]. Furthermore, we report evidence that H type 3 is a most probable pluripotent glycan marker responsible for this lectin probe.

Materials and Methods

Cell

Human endometrium (UtE1104), amnion (AM936EP), and placental artery endothelium (PAE551) were collected by scraping tissues from surgical specimens as a therapy under signed informed consent, with ethical approval of the Institutional Review Board of the National Institute for Child Health and Development of Japan [11]. All experiments handling human cells and tissues were performed in line with the tenets of the Declaration of Helsinki. Endometrium, amnion, and placental artery endothelium cell lines were independently established. However, their actual origins remain to be clarified. Human iPS cells (MRC5-iPS, AM-iPS, UtE-iPS, and PAE-iPS) were then generated via procedures described by Yamanaka and colleagues [12, 13] and others [7, 14–16]. iPS201B7 and iPS253G1 human iPS cells were cultured in DMEM/Ham's F-12 medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 20% Knockout serum replacement (KSR) (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), minimal essential medium (MEM) nonessential amino acids (Invitrogen), and 10 ng/ml recombinant human basic fibroblast growth factor (Wako) on mitomycin C-treated mouse embryo fibroblast feeder cells. Human iPS cells (TIG/MKOS nos. 19, 56, and 106) were generated through reprogramming by Sendai virus infection-mediated expression of OCT4, SOX2, KLF4, and c-MYC as previously described [17]. The production of iPS cells was confirmed by immunofluorescence staining using established stem cell markers (Nanog, Oct3/4, SSEA4, and TRA-1-60) and analysis of the capacity for teratoma formation (supplemental online Fig. 1). Human ES cells (KhES1, KhES2, KhES3, and HUES1) were obtained and maintained as previously described [18, 19]. A list of the cell lines used in this study and their associated characteristics is given in supplemental online Table 1.

Immunoprecipitation of Human Podocalyxin

Hydrophilic fractions of cells were prepared using CelLytic MEM Protein Extraction (Sigma-Aldrich) in accordance with the manufacturer's procedures [14, 20]. Dynabeads M-280 streptavidin (Invitrogen; 10 μl) immobilized with biotinylated anti-podocalyxin polyclonal antibody (pAb) (1 μg) were incubated with hydrophilic fractions at 4°C overnight with agitation. After the beads were washed with 200 μl of phosphate-buffered saline (10 mM phosphate-buffered saline, pH 7.4, 140 mM NaCl, 2.7 mM KCl) containing 1% Triton X-100 (PBST) three times, bound podocalyxin was eluted with 50 μl of Tris-buffered saline (TBS) containing 0.2% SDS at 95°C for 10 minutes.

Western and Lectin Blotting

One microgram of the hydrophilic fractions or 5 μl of the immunoprecipitated samples described above were electrophoresed under reducing conditions on 5%–20% polyacrylamide gel (Dream Realization & Communication, Tokyo, Japan, http://www.drc2002.com/index.html; catalog no. NTH-676HP). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with BlockAce (Funakoshi, Tokyo, Japan, http://www.funakoshi.co.jp; catalog no. BUF029), the membrane was incubated with horseradish peroxidase (HRP)-conjugated rBC2LCN (1 μg/ml) or goat anti-podocalyxin pAb (0.2 mg/ml; R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com; catalog no. AF1658) followed by HRP-conjugated donkey anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com; catalog no. 705-035-003). Silver staining of the gel was also performed using a silver staining kit (Wako Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english; catalog no. 299-58901). The HRP labeling of rBC2LCN was performed using a peroxidase labeling kit (Dojindo Molecular Technologies Inc., Gaithersburg, MD, http://www.dojindo.com; LK11). The immunoprecipitated podocalyxin (10 μl) was heat-denatured at 95°C for 5 minutes and treated with 1 μl of Peptide:*N*-glycosidase F (PNGase F; ProZyme, Hayward, CA, http://www.prozyme.com; catalog no. GKE-5003) at 37°C overnight to remove *N*-glycans. Selective β-elimination of *O*-glycans from podocalyxin was performed by incubating the Western blot with 0.05 M NaOH for 16 hours at 40°C before probing with HRP-labeled rBC2LCN as described [21, 22].

Production of High-Density Lectin Microarray

The high-density lectin microarray immobilizing 96 lectins including rBC2LCN was prepared as previously described [7, 23, 24]. Each lectin was spotted at 0.5 mg/ml in triplicate (supplemental online Fig. 2).

Antibody-Overlay Lectin Microarray

Immunoprecipitated samples were prepared from mouse embryonic fibroblasts (MEFs) (no. 1), somatic cells (nos. 2–15), iPS cells

(nos. 16–153), and ES cells (nos. 154–168), treated with *Arthrobacter ureafaciens* sialidase (Roche, Indianapolis, IN, http://www.roche.com; catalog no. 10269611001, 1 μl) in PBST at 37°C overnight and incubated with high-density lectin microarray at 20°C overnight (supplemental online Fig. 3). After washing with probing buffer (25 mM Tris-HCl, pH 7.5, 140 mM NaCl [TBS], 2.7 mM KCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1% Triton X-100), the array was blocked with 60 μl of rabbit normal IgG (50 μg/ml) at 20°C for 1 hour. After washing again with probing buffer, the array was reacted with biotinylated goat anti-podocalyxin pAb (R&D; catalog no. AF1658) for 1 hour at 20°C. After a further wash with probing buffer, bound anti-podocalyxin pAb was detected with 1 μg/ml of Cy3-labeled streptavidin at 20°C for 30 minutes. After washing with probing buffer, fluorescence images were acquired using an evanescent field-activated fluorescence scanner (GlycoStation Reader 1200; GP BioSciences, Sapporo, Japan, http://www.gpbio.jp/english/index.html). The fluorescence signal of each spot was quantified using Array Pro Analyzer ver.4.5 (Media Cybernetics, Bethesda, MD, http://www.mediacy.com), and the background value was subtracted. The lectin signals of triplicate spots were averaged and normalized to the mean value of 96 lectins immobilized on the array to adjust the data from each microarray to account for possible systematic variation [20, 25].

Gene Expression Analysis

Total RNA was extracted from each cell sample using ISOGEN (Nippon Gene, Tokyo, Japan, http://www.nippongene.com). Global gene expression patterns were monitored using whole human genome microarray chips (G4112F; Agilent Technologies, Palo Alto, CA, http://www.agilent.com) with one-color (Cyanine 3) dye. Hybridization was determined with a G2505C microarray scanner system (Agilent). The data were analyzed using GeneSpring GX12.0 software (Agilent). Each chip was normalized to the 75th percentile of measurement taken from the chip.

Frontal Affinity Chromatography

The principle and protocol of frontal affinity chromatography (FAC) have been described previously [26, 27]. rBC2LCN was immobilized onto NHS-activated Sepharose 4FF (GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com), packed into a miniature column (inner diameter, 2 mm; length, 10 mm; bed volume, 31.4 μl; Shimadzu, Kyoto, Japan, http://www.shimadzu.com), and connected to a high-performance liquid chromatograph (Shimadzu). Pyridylaminated (PA) glycans prepared from human iPS cells (201B7) were injected into the column. The elution profile was then detected by fluorescence (excitation, 285 nm; emission, 350 nm). The elution front of PA glycan relative to that of negative control PA glycan (Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc-PA), referred to as V-V_0, was then determined. Woolf-Hofstee plots were constructed using the V-V_0 values. The intercept and slope of the fitted line represent B_t (nmol) and $-K_d$ (μM), respectively. Analysis of concentration dependence was performed using globo H (Fucα1–2Galβ1–3GalNAcβ1–3Galα1–4Galβ1–4Glc)-*p*-nitrophenol.

RESULTS

Podocalyxin Is a Candidate Ligand of rBC2LCN as Revealed by DNA Microarray

Using the high-density lectin microarray, we have previously shown that a probe lectin, rBC2LCN, exhibits rigorous specificity to undifferentiated human iPS cells and ES cells, but not at all to differentiated somatic cells [7]. Therefore, identification of glycoprotein ligands of rBC2LCN is critical to determine the molecular mechanism of undifferentiation from both developmental and glycobiological viewpoints. As a practical approach, we focused our attention to a candidate glycoprotein, podocalyxin, with respect to the following criteria: (a) highly expressed in human iPS cells and ES cells; (b) correlated with Nanog expression (pluripotency marker); (c) localized at the cell surface; (d) heavily glycosylated; and (e) antibodies used for immunoprecipitation are commercially available. In gene expression analysis of human iPS cells, ES cells, and somatic cells using DNA microarray, the podocalyxin gene was found to be highly expressed in all of the human iPS cells and ES cells (criteria a and b) (Fig. 1). High expression was also detected in one of the somatic cells derived from placental artery endothelial (PAE) and low but significant expression in other three somatic cells of MRC5, amniotic mesodermal (AM), and uterine endometrium (UtE) at the transcription level. Human podocalyxin is a type 1 membrane protein consisting of 528 amino acids, with criterion c thus satisfied given that the protein is localized at the cell surface [28]. The extracellular domain of podocalyxin has a mucin domain with a high serine/threonine content for putative *O*-glycosylation modifications. Podocalyxin also has five potential *N*-linked glycosylation sites and three putative glycosaminoglycan sites. Indeed, the apparent molecular weight has been reported to be ~200 kDa in embryonic carcinoma cells [29], despite the calculated molecular weight of 55 kDa, demonstrating that human podocalyxin is heavily glycosylated (criterion d). Various types of polyclonal and monoclonal antibodies raised against the extracellular and the intracellular domains of human podocalyxin are commercially available (criterion e).

High Molecular Weight Proteins of >240 kDa Are the Glycoprotein Ligands of rBC2LCN in Human iPS Cells and ES Cells

To search glycoprotein ligands of rBC2LCN in human iPS cells and ES cells, hydrophilic fractions of human iPS cells and ES cells in addition to mouse feeder cells and somatic cells were first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted with HRP-conjugated rBC2LCN. Representative iPS cells prepared from various tissue types (MRC5-iPS25 [P22] [18], AM-iPS3 [P7] [29], UtE-iPSA04 [P27] [71], PAEiPS01 [P26] [95], TIG/MKOS19 [P37] [141], HiPS201B7 [P45] [123], hiPS253G1 [P32] [128]) and ES cell lines (KhES-1 [P26] [156], KhES-2 [P31] [159], KhES-3 [P28] [160], HUES1 [163]) were randomly selected among 138 types of iPS cells and 15 types of ES cells (supplemental online Table 1) and used for this analysis. As shown in Figure 2, rBC2LCN bound to all of the undifferentiated iPS cells and ES cells tested, but not at all to differentiated mouse feeder cells (MEFs) (no. 1) and somatic cells of the iPS origin (MRC5 [no. 2], AM936EP [no. 5], UtE1104 [no. 9], PAE551Ar [P16] [no. 11], and h-Fib [no. 13]), supporting the previous report that rBC2LCN is highly specific to undifferentiated cells [7]. In iPS cells and ES cells, strong and diffuse protein bands were detected at a high molecular weight of >240 kDa, whereas weaker bands were also detected from 70 to 140 kDa, indicating that other glycoprotein ligands of rBC2LCN are also present in human iPS and ES cells.

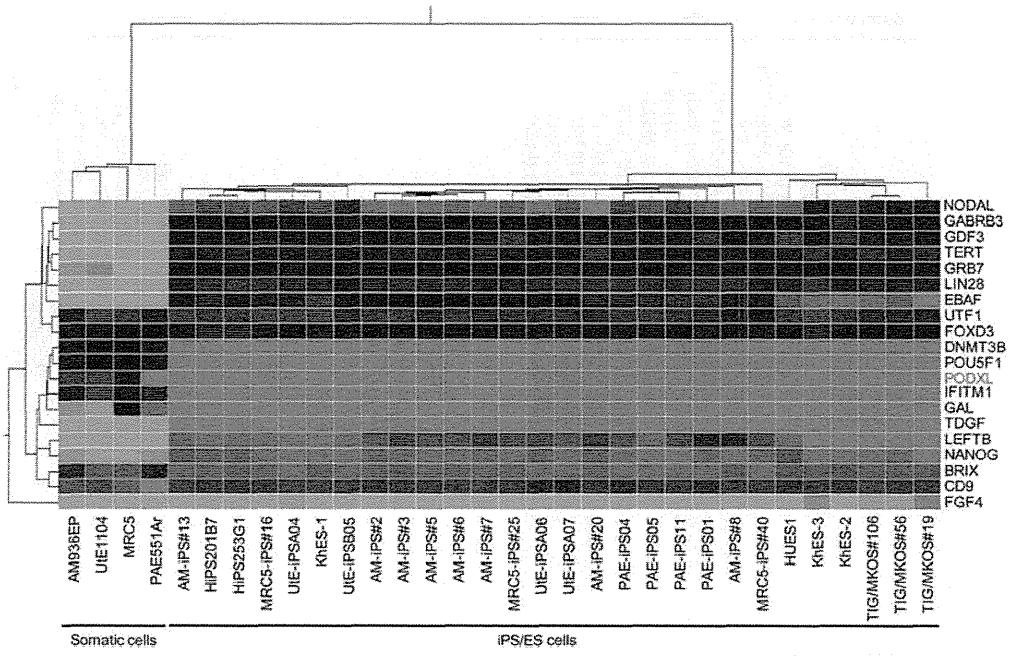

Figure 1. Clustering of human stem cell markers versus cell lines. The heat map shows a two-way cluster analysis carried out on the data of the cell lines listed in supplemental online Table 1. The data were obtained as averages of multiple cell samples. Levels of gene expression are indicated by color changes from red (high expression levels) to green (low expression levels). Podocalyxin expression is indicated as "PODXL" in red. Abbreviations: ES, embryonic stem; iPS, induced pluripotent stem.

Podocalyxin Is a Glycoprotein Ligand of rBC2LCN

The molecular nature of the rBC2LCN-positive bands such as a high molecular weight and high expression in human iPS cells and ES cells strongly suggested that podocalyxin is a glycoprotein ligand of rBC2LCN. Streptavidin-coated magnetic beads immobilized with biotinylated goat anti-podocalyxin pAb were incubated overnight at 4°C with hydrophilic fractions of iPS cells. After extensive washing of the beads, bound fractions were eluted with TBS containing 0.2% SDS at 95°C for 10 minutes. The elution fractions were then separated by SDS-PAGE and blotted with goat anti-podocalyxin pAb followed by HRP-labeled donkey anti-goat IgG. As shown in Figure 3 (bottom panel), podocalyxin could be immunologically detected as a diffuse high molecular weight band at >240 kDa in the immunoprecipitates from human iPS cells and ES cells, but not those from mouse feeder cells and somatic cells. Because the calculated molecular weight of podocalyxin is 55 kDa, it is evident that podocalyxin, expressed in human iPS cells and ES cells, is heavily glycosylated. Indeed, rBC2LCN exhibited strong binding to the immunoprecipitated podocalyxin as a diffuse protein band at >240 kDa in human iPS cells and ES cells (Fig. 3, top panel). Stronger staining was observed with rBC2LCN relative to anti-podocalyxin pAb. The rBC2LCN reactivity varied among the cell types of iPS cells and ES cells. Stronger signals were observed for UtE-iPS A04 (P27) (71) and PAE-iPS01 (P26) (95), whereas weaker signals were detected for TIG/MKOS19 (P37) (141), iPS201B7 (P45) (123), and iPS253G1 (P32) (128). Although the podocalyxin gene was detected in PAE551Ar, the corresponding podocalyxin protein was not detected in the anti-podocalyxin immunoprecipitates (Fig. 3), indicating that the podocalyxin expression is restricted to undifferentiated cells at the translation level among the cell types used in this study (supplemental online Table 1). It should be noted that podocalyxin could be precipitated from hydrophilic but not hydrophobic fractions (data not shown), even though podocalyxin is basically a transmembrane protein. This can be attributed, at least in part, to the highly hydrophilic nature of podocalyxin, which is heavily glycosylated. Altogether, these results clearly demonstrate that podocalyxin is a glycoprotein ligand of rBC2LCN, although the possibility that other glycoprotein ligands with >240 kDa are included remains.

Podocalyxin Is a Glycoprotein Ligand of rBC2LCN Common to Human iPS Cells and ES Cells Revealed by Antibody-Overlay Lectin Microarray

To examine whether rBC2LCN binds to human podocalyxin derived from human pluripotent stem cells of more than 100 samples in a high-throughput manner, we applied the recently developed high-throughput technology called antibody-overlay lectin microarray, which allows interaction analysis between immobilized lectins and a nanogram order of target samples [10]. A schematic representation of the technique is shown in supplemental online Figure 3. The human podocalyxin immunoprecipitated from human iPS cells and ES cells was reacted with rBC2LCN immobilized on a glass slide [7, 23]. After blocking of nonspecific binding sites with normal rabbit IgG, bound podocalyxin was selectively visualized with biotinylated anti-podocalyxin pAb followed by Cy3-labeled streptavidin, according to the established protocol [10]. The binding signals were detected and quantified using an evanescent-field fluorescence-assisted scanner as described [23]. To further confirm this, the immunoprecipitated podocalyxin was pretreated with *A. ureafaciens* sialidase before application to the lectin microarray, because this treatment was found to enhance the interaction between podocalyxin and the immobilized rBC2LCN. This could be explained in part by reduced electric repulsion caused by the strong negative

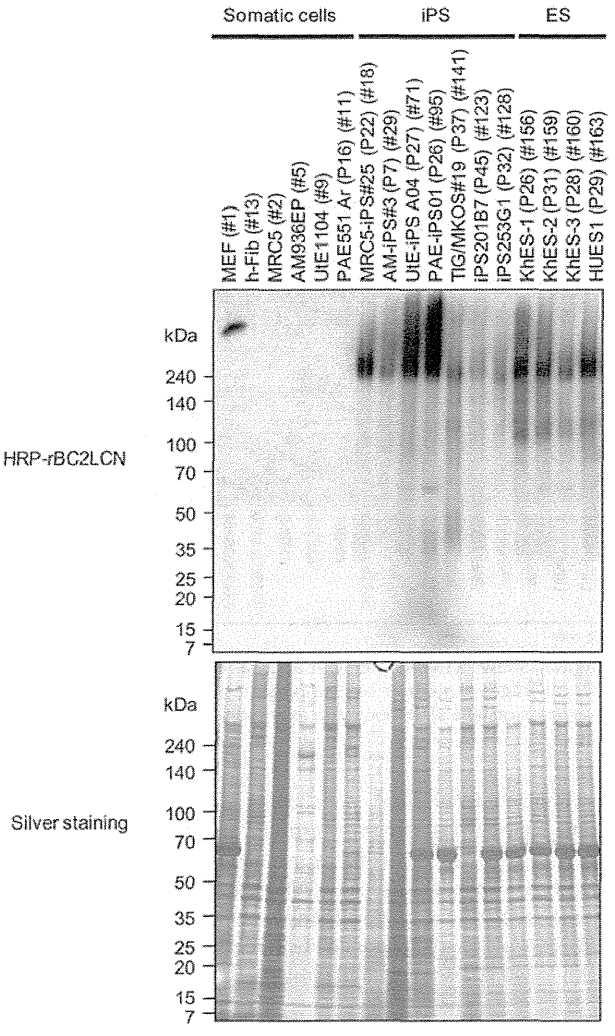

Figure 2. High molecular weight proteins of >240 kDa are glycoprotein ligands of rBC2LCN in human iPS cells and ES cells. One microgram of hydrophilic fractions of somatic cells, iPS cells, and ES cells was run on 5%–20% acrylamide gel under reducing conditions, electroblotted onto polyvinylidene difluoride membrane, and stained with 1 μg/ml of HRP-conjugated rBC2LCN (top panel). Silver staining was also performed (bottom panel). Abbreviations: ES, embryonic stem; HRP, horseradish peroxidase; iPS, induced pluripotent stem; MEF, mouse embryonic fibroblasts.

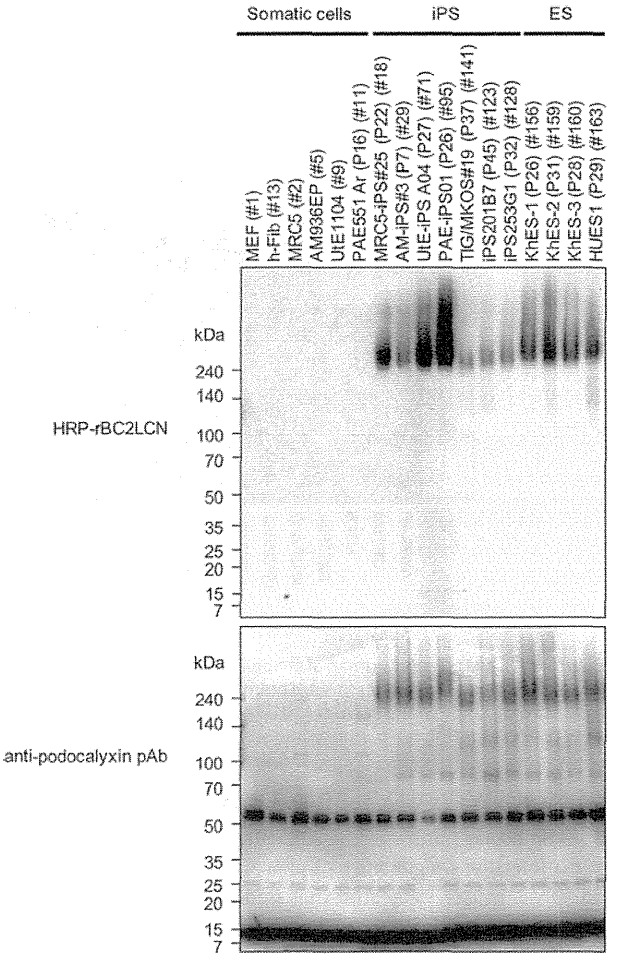

Figure 3. Podocalyxin is a glycoprotein ligand of rBC2LCN. Dynabeads M-280 streptavidin (10 μl) immobilized with biotinylated anti-podocalyxin pAb (1 μg) was incubated with hydrophilic fractions of cells at 4°C overnight with agitation. After washing the beads with 200 μl of PBST three times, bound podocalyxin was eluted with 50 μl of TBS containing 0.2% SDS at 95°C for 10 minutes. Coeluted biotinylated anti-podocalyxin pAb was partially depleted with Dynabeads M-280 streptavidin (15 μl) at room temperature for 1 hour with agitation. The eluted fractions were run on 5%–20% acrylamide gel under reducing condition and transferred to polyvinylidene difluoride membrane. The membrane was stained with either HRP-conjugated rBC2LCN (1 μg/ml, top panel) or goat anti-podocalyxin pAb (0.1 μg/ml) followed by HRP-conjugated donkey anti-goat IgG (×10,000, bottom panel). The staining was performed in different membranes. Both 55- and 10-kDa bands are nonspecific, since both bands could be detected in the control immunoprecipitated samples. The 55-kDa band might be the heavy chain of goat anti-podocalyxin pAb used for the immunoprecipitation of podocalyxin, whereas the identity of the 10-kDa band is unknown. Abbreviations: ES, embryonic stem; HRP, horseradish peroxidase; iPS, induced pluripotent stem; MEF, mouse embryonic fibroblasts; pAb, polyclonal antibody.

charge of the heavily sialylated podocalyxin [30]. Using the advanced high-throughput technology, a series of cell samples including 138 types of human iPS cells prepared from six different origins with various passages (16–153 in supplemental online Table 1) and 15 types of human ES cells (154–168) were analyzed [20]. For reference (negative control), mouse feeder cells (MEFs) (1) and differentiated somatic cells of the iPS origin (2–15) were also examined. Figure 4A shows the results of rBC2LCN binding for representative samples, that is, MEFs (1), fibroblasts (166), iPS cells (115), and ES cells (154), whereas Figure 4B provides a bar graph representation of the total analysis. It was verified unambiguously that all of the iPS cells and ES cells examined bound to rBC2LCN, although the binding degrees were significantly varied. On the other hand, no detectable signal was observed for rBC2LCN binding to feeder (1) or somatic cells (2–15) as expected, since podocalyxin was not immunoprecipitated from these cell samples (Fig. 3). These results confirm the fact that podocalyxin is a universal glycoprotein ligand of rBC2LCN in human undifferentiated pluripotent cells.

Glycan Ligands on Podocalyxin Recognized by rBC2LCN Are *O*-Glycans

Having demonstrated that podocalyxin is a substantial glycoprotein ligand of rBC2LCN both in iPS cells and ES cells, we next

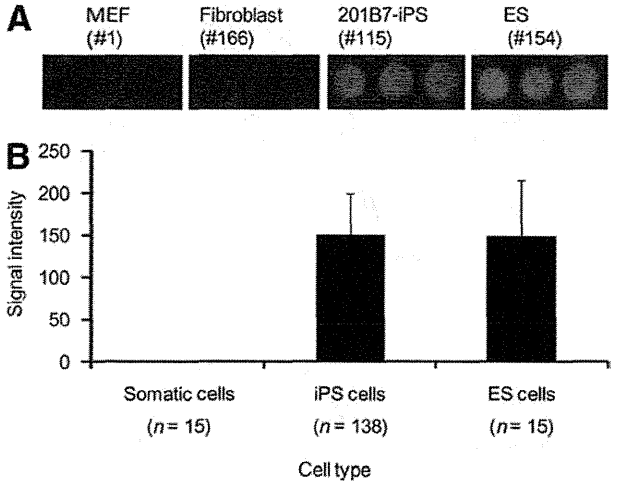

Figure 4. Podocalyxin is a glycoprotein ligand common to human iPS cells and ES cells. Podocalyxin was immunoprecipitated from hydrophobic fractions with streptavidin-coated magnetic beads immobilizing biotinylated anti-podocalyxin polyclonal antibody (pAb). The elution fraction was pretreated with *A. ureafaciens* sialidase and incubated with high-density lectin microarray containing rBC2LCN. After blocking with rabbit IgG, the array was incubated with biotinylated anti-podocalyxin pAb followed by Cy3-labeled streptavidin and scanned with an evanescent-field fluorescence scanner. Representative binding images obtained with a 120× gain on triplicate spots of rBC2LCN are shown in **(A)**. The fluorescence signal of each spot was quantified using Array Pro Analyzer version 4.5, and the background value was subtracted. The lectin signals of triplicate spots were averaged and normalized to the mean value of 96 lectins immobilized on the array. The data are shown as the averages ± S.D. of the binding of rBC2LCN to podocalyxin immunoprecipitated from somatic cells (n = 14; 2–15 in supplemental online Table 1), iPS cells (n = 138; 16–153), and ES cells (n = 15; 154–168) **(B)**. Abbreviations: ES, embryonic stem; iPS, induced pluripotent stem; MEF, mouse embryonic fibroblasts.

analyzed the glycan structures on podocalyxin and the classes of glycans to which rBC2LCN binds. For this purpose, we first performed antibody-overlay lectin microarray assisted with enzyme treatments (supplemental online Fig. 4). Since podocalyxin has been reported to contain *N*- and *O*-glycans, and keratan sulfate glycosaminoglycans [4, 28, 31–33], the protein was digested with PNGase F, an amidase that cleaves *N*-glycans between the innermost GlcNAc and asparagine residues of *N*-linked glycoproteins, and with keratanase II from *Bacillus* sp. Ks36, an endo-β-*N*-acetylglycosaminidase that hydrolyzes keratan sulfate between the 4GlcNAcβ1–3Galβ1 structure [34]. Before the enzyme treatments, podocalyxin was heat-denatured and treated with *A. ureafaciens* sialidase to increase the enzyme susceptibility. Podocalyxin immunoprecipitated from TIG/MKOS19 (P50) chosen as a representative iPS cell line was treated with either sialidase, sialidase and PNGase F, or sialidase and keratanase II and incubated with high-density lectin microarray. Bound podocalyxin was analyzed by the antibody-overlay method as described above. As shown in supplemental online Figure 4, the signals for SNA (*Sambucus nigra* agglutinin), an α2–6Sia-binding lectin, were significantly decreased after the sialidase treatment, demonstrating that podocalyxin is α2–6sialylated. The residual signals are attributable to either the incomplete sialidase digestion or the binding to asialo *N*-glycans, because SNA also shows weak but significant binding to LacNAc (Galβ1–4GlcNAc) [35]. With the sialidase and PNGase F double treatments, the binding of a high-mannose-type *N*-linked glycan-binding lectin (recombinant griffithsin [rGRFT]) was also decreased. This indicates that podocalyxin carries high-mannose type *N*-linked glycans [32]. The binding of *Psathyrella velutina* lectin (PVL) with specificity to nonsubstituted βGlcNAc and 6-*O*-substituted βGlcNAc, including 6S-GlcNAc at the nonreducing end [36, 37] was decreased upon double treatments with sialidase and keratanase II. This observation supports a previous report that podocalyxin contains keratan sulfate, which is susceptible to keratanase II treatment [4]. The results also suggest that keratan sulfate is largely attributed to *O*-glycans, since the PVL signal was not susceptible to PNGase F treatment. In contrast, the signals of an *O*-glycan binder (*Amaranthus caudatus* lectin [ACA]) were increased after sialidase treatment and further enhanced with sialidase and keratanase II double treatments. Increased signals can be explained by the increased accessibility of this *O*-glycan binding lectin caused by the decreased charge repulsion and/or steric hindrance caused by heavy sialylation and sulfation of podocalyxin. Furthermore, significant signals of the α1–2fucose-binding lectin MCA could be observed, which were also increased upon enzyme treatments. Similarly, the signals of rBC2LCN were increased after sialidase treatment and further enhanced with the sialidase and keratanase II double treatment, leading to the hypothesis that the major carbohydrate antigens of rBC2LCN are *O*-glycans.

To prove this hypothesis, we further performed a lectin blotting experiment. The podocalyxin immunoprecipitated from iPS cells (iPS201B7 [P45], 123) and ES cells (KhES-1 [P26], 156) was treated with or without PNGase F, run on SDS-PAGE, and blotted onto PVDF membrane. The membrane was then treated with or without 0.05 M NaOH at 40°C for 16 h to remove *O*-glycans by β-elimination [21, 22] and analyzed by lectin blotting using HRP-conjugated rBC2LCN. As shown in Figure 5, most rBC2LCN signals were still left upon PNGase F treatment, whereas the alkaline hydrolysis greatly reduced the rBC2LCN binding, demonstrating that the carbohydrate antigens of rBC2LCN are expressed on *O*-glycans of podocalyxin prepared from human iPS cells and ES cells. Since the alkaline treatment gave essentially no effect on the rBC2LCN binding to a positive control neoglycoprotein (Fucα1–2Galβ1–3GlcNAcβ1–3Galβ1–4Glc-BSA), the reduced staining is not due to the loss of the blotted proteins.

rBC2LCN Recognizes an *O*-Glycan Containing an H Type 3 Isolated From iPS Cells

Recently, we performed quantitative glycome analysis targeting both *N*- and *O*-glycans of undifferentiated iPS cells (201B7) and differentiated human dermal fibroblasts by a glycosidase-assisted high-performance liquid chromatography (HPLC) method combined with mass spectrometry [38]. Among the 37 types of *N*-glycans and 10 types of *O*-glycans identified from iPS cells, one *O*-glycan with an m/z value of 973.4 $[M+H]^+$ containing an H type 3 (Fucα1–2Galβ1–3GalNAc) was isolated, of which structure was identified to be Fucα1–2Galβ1–3(Galβ1–3GlcNAcβ1–6)GalNAc-PA by HPLC mapping assisted with matrix-assisted laser desorption ionization time-of-flight mass spectrometry and exoglycosidase digestion analyses [38], where "PA" represents a reducing terminal pyridylamino group. On the other hand, no glycan containing H type 1 was detected. Thus, we determined the association constant between rBC2LCN and this *O*-glycan carrying an H type 3 structure (hereafter designated glycan *a*)